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Running title: Surface electrostatics and H/D exchange in proteins

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Abstract

This paper combines two techniques—mass spectrometry and protein charge ladders—to examine the relationship between the surface charge and hydrophobicity of a protein (bovine carbonic anhydrase II; BCA II) and its rate of amide hydrogen-deuterium (H/D) exchange. Mass spectrometric analysis indicated that the sequential acetylation of surface lysine-ε-NH_3^+ groups—a type of modification that increases the net negative charge and hydrophobicity of the surface of BCA II without affecting its 2° or 3° structure—resulted in a linear increase in the total number of backbone amide hydrogen that are protected from exchange with solvent (2 h, pD 7.4, 15 ºC). Each successive acetylation produced BCA II proteins with one additional hydrogen protected after two hours in deuterated buffer (pD 7.4, 15 ºC). NMR spectroscopy demonstrated that these protected hydrogen atoms were *not* located on the side chain of the acetylated lysine residues (i.e., lys-ε-$NHCOCH_3$). The decrease in rate of exchange associated with acetylation paralleled a *decrease* in thermostability: the most slowly exchanging rungs were the least thermostable (as measured by differential scanning calorimetry). The fact that the rates of H/D exchange were similar for perbutyrated BCA II (e.g., [lys-ε-$NHCO(CH_2)_2CH_3]_{18}$) and peracetylated BCA II (e.g., [lys-ε-$NHCOCH_3]_{18}$) suggests that the charge is more important than the hydrophobicity of surface groups in determining the rate of H/D exchange. These kinetic electrostatic effects could complicate the interpretation of experiments in which H/D exchange methods are used to probe the structural effects of non-isoelectric perturbations to proteins (i.e., phosphorylation, acetylation, or the binding of the protein to an oligonucleotide or another charged ligand or protein).

Key words: amide H/D exchange, lysine acetylation, mass spectrometry, protein folding, carbonic anhydrase II, protein charge ladder, hydrogen/deuterium, electrostatic potential.

Introduction

We wished to determine how the surface charge and hydrophobicity of a folded protein affects the rate at which it exchanges amide N-H hydrogen with buffer, and have measured the rate of hydrogen-deuterium (H/D) exchange of the rungs (successively acylated sets of proteins) of two protein charge ladders[1-5] with electro-spray ionization mass spectrometry (ESI-MS). A "protein charge ladder" is a mixture of charge isomers generated by the modification of the functional groups of a protein. The charge ladders we used were prepared by sequentially acylating all 18 lysine-ε-NH_3^+ of bovine carbonic anhydrase II[6] (BCA II) with acetic or butyric anhydride to yield lysine-ε-$NHCOCH_3$ and lysine-ε-$NHCO(CH_2)_2CH_3$.

The isoelectric point (pI) of BCA II is ~ 5.9. Previous experiments at pH 8.4 have shown that each acetylation increases the net negative charge (Z_o) of BCA II by approximately 0.9 units. The difference between $\Delta Z = -0.9$ and the value of -1.0 that might be expected for -NH_3^+ → -$NHCOCH_3$ can be explained by charge regulation [7] (e.g., the electrostatic effect of acylating -ε-NH_3^+ is not limited to the ε-nitrogen that is modified). Solvent ions, for example, will reorganize around the ε-nitrogen, and the values of pK_a of nearby ionizable groups will adjust to the new electrostatic environment that results from neutralization of the lysine ε-NH_3^+ group. The BCA II charge ladder contains 19 charge isomers or "rungs," and therefore spans approximately 16 units of charge. The acetylation of all 18 lysine residues (*peracetylation*) does not change the structure of this thermostable zinc protein (as measured previously by circular dichroism[3] and X-ray crystallography[8]).

Mass spectrometry established a linear relationship between the net negative charge of folded BCA II (e.g., the number of acylations) and the number of hydrogens that do not exchange with solvent after a 2 h incubation in deuterated buffer (we say these hydrogen are *protected* from exchange). The acetylation of each lysine, for example, generated approximately one additional hydrogen that was protected from H/D exchange after 2 h (at 15 °C, pD 7.4). Multi-dimensional Nuclear Magnetic Resonance (NMR) spectroscopy demonstrated that the additional protected hydrogen atoms were *not* located on the lysine-acetyl side chains, but were present in amide NH groups located on the backbone of the polypeptide. Although the most negatively charged rungs of the ladder

had the slowest rates of global[9] H/D exchange, an analysis with differential scanning calorimetry showed that these rungs also had lower conformational stability than the lower rungs.

Hydrogen Exchange as a Tool for Studying the Structure and Folding of Proteins. The rate at which a protein exchanges its backbone amide hydrogens with tritium or deuterium in buffer has been used for nearly 60 years[10, 11] to study the structure [12, 13], folding [14], and conformational stability[15-17] of proteins. In fact, the first measurements of H/D exchange were not made with any form of spectroscopy, but rather by determining the density of H_2O droplets after the addition of deuterated protein (that had been flash-frozen as a function of time in D_2O and then dried under vacuum with P_2O_5).[11] The utility of hydrogen exchange in protein biochemistry is based upon the generally observed correlation between the rate of amide hydrogen exchange and i) the rate of protein folding, ii) the local structure surrounding a backbone amide, and iii) the conformational stability of the folded protein.[17, 18] In spite of the historic and now widespread use of hydrogen exchange in structural biology and biochemistry—and in spite of all that is known about the processes of H/D exchange in proteins—the reasons for why many amide hydrogen atoms are slow to exchange in *folded* polypeptides (and other types organic molecules for that matter [19-21]) are still not completely understood [22] (this matter is discussed further below).

The exchange of amide hydrogens with aqueous solvent is catalyzed by both acid and base, and the minimum rate of exchange for an unstructured polypeptide occurs at ~ pH 2.5.[23] Above pH 4, the primary catalyst for amide hydrogen exchange is hydroxide [24] (the pK_a of the backbone amide in an unstructured polypeptide is ~ 15); below pH 4, the exchange is catalyzed by hydronium. In the case of an unstructured polypeptide, the exchange of amide hydrogen with solvent is fast: it occurs in milliseconds to seconds at pH 7 and room temperature.[25] With a folded or structured protein, however, the rate of exchange can be slower by factors of 10^8 (at pH 7 and room temperature).[26, 27]

A simple kinetic model, developed by Linderstrøm-Lang, has been used for decades to understand the kinetics of amide hydrogen exchange in folded proteins.[11, 28]

This model (summarized in Equation 1) involves a transition between two states: "open" and "closed". Hydrogen exchange occurs in the "open" state and not in the "closed" state.

$$NH_{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} NH_{open} \xrightarrow{k_{int}} ND_{open} \; ; K_{op} = k_{op}/k_{cl} \qquad (1)$$

In Equation 1, k_{int} refers to the rate constant for the exchange of an amide in an unstructured polypeptide (i.e., the *intrinsic* rate of exchange); k_{cl} refers to the rate constant for a closing reaction (e.g., refolding or a change in conformation). The *intrinsic* rate of hydrogen exchange for all 20 amino acids have been characterized (as a function of temperature and pH) using model peptides.[29, 30]

The reaction scheme in (1) can occur at two extremes: i) $k_{cl} >> k_{int}$; that is, the closing reaction (such as folding or a change in conformation) is much faster than the intrinsic rate of exchange; and: ii) $k_{cl} << k_{int}$. These two conditions, described by equations 2 and 3, are termed EX2 (i.e., k_{obs} depends on two terms) and EX1 (k_{obs} depends on one term). [28, 31, 32]

$$\text{EX2 (i.e., when } k_{cl} >> k_{int}\text{): } k_{obs} = K_{op} k_{int} \qquad (2)$$

$$\text{EX1 (i.e., when } k_{cl} << k_{int}\text{): } k_{obs} = k_{op} \qquad (3)$$

The two most widely accepted theories for understanding how various amides undergo hydrogen exchange in proteins are known as "local unfolding" [18, 24] and "solvent penetration". [33, 34] These models theorize that amide hydrogens exchange slowly in folded proteins because of hydrogen bonding (the *local unfolding* model) or burial in the protein and physical protection from contact with solvent and catalysts (the *solvent penetration* model). The local unfolding model postulates that local fluctuations in protein structure permit exchange by separating NH and CO groups (by ≥ 5Å) that are H-bonded in α-helices or β-sheets[18]; the solvent penetration model postulates that water or a catalyst permeate the protein (without its unfolding, *per se*). These two theories are not mutually exclusive; Dill has reviewed data in support of each.[35]

The results of studies of hydrogen exchange on small-molecule amides and model peptides have made it reasonable, in our opinion (and in the opinion of others[20, 36, 37]), to

suspect that amides in *closed* configurations (Eq. 1) are not simply protected from hydrogen exchange because of hydrogen bonding and solvent accessibility alone and that electrostatic effects can greatly affect, in the some cases, the rate of H/D exchange. Hydrogen exchange studies of model amides (i.e., N-methyllauramide and N-methylbutyramide) in the presence of cationic, neutral and anionic micelles have suggested that the electrostatic environment of an amide affects its rate of hydrogen exchange.[20] For example, the rate constant for base-catalyzed exchange (k_{OH}) of model amides decreased by factors of 2500 in the presence of negatively charged micelles, whereas k_H increased 100 fold.[20] Decreases in k_{OH} were not observed in the presence of neutral or cationic micelles (although a 30-fold decrease in k_H was observed in the presence of cationic micelles). The relative rates of hydrogen exchange for diketopiperazine and 2-piperidone (the mono-amide analog of diketopiperazine) are also interesting: the k_{OH} of diketopiperazine is ~ 740 times greater than for 2-piperidone.[36]

Confounding matters even further, the pioneering studies that used model amino acids and oligopeptides to determine the effect of primary structure on rates of amide hydrogen exchange were performed at high concentrations of salt (i.e., 0.5 M KCl) in order to "shield possible charge effects".[30 38] We believe, however, that understanding electrostatic effects on amide hydrogen exchange in proteins is necessary to understand them mechanistically and to understand what they reveal—and what they do not necessarily reveal—of the structure and folding of proteins.

Finally, there are examples of amino acids in proteins (i.e., lysozyme and rubredoxin) whose backbone amides are exposed to solvent and *not* H-bonded but that do, nevertheless, undergo H/D exchange at rates that are up to a billion-fold slower than the rate of a corresponding model oligopeptides; these surface residues (i.e., Val 38 in *Pyrococcus furiousus* rubredoxin [22]) exchange as if they were in the hydrophobic core of the protein or engaged in strong H-bonds. A series of recent papers have suggested that variations in the electrostatic potential across the surface of proteins such as rubredoxin are likely to explain why such solvent-exposed and non-H-bonded amino acids exchange so slowly [22, 39, 40] (and why other amides exchange more rapidly than would be expected based upon their deep burial from solvent and H-bonding interactions [41]).

Using Protein Charge Ladders and Mass Spectrometry to Measure H/D Exchange in Proteins. There are few experimental tools available with which to investigate how the electrostatic environment of amides in folded proteins affects their rates of H/D exchange. Much of the previous work investigating electrostatic effects in the hydrogen-exchange rate of folded proteins has compared the rate of exchange at different values of pH [42, 43] or ionic strength.[44, 45] In these types of experiments, any change in the rate of exchange of hydrogen of a protein (e.g., k_{obs} in Eq. 1) that occurs with pH or ionic strength is compared to changes in the intrinsic rate of exchange (e.g., k_{int} in Eq. 1); it is difficult, however, to determine the origin of effects observed in this sort of experiment. A change in pH, *inter alia*, can change the structure of a protein, in addition to changing its net charge[46, 47] (serum albumin, for example, undergoes distinct changes in conformation at pH 2.7, 4.3, 7.0, ~8 and ~10).[48]

A protein charge ladder provides a straightforward and internally consistent tool with which to study kinetic electrostatic effects in the hydrogen exchange of a folded protein. The charge ladder of BCA II represents a set of proteins that have different values of net charge, but similar structures (and identical amino acid sequences).[8] The rates of H/D exchange of all 19 rungs can be measured simultaneously using mass spectrometry; each charge isomer is, therefore, measured under conditions of identical medium pH, ionic strength, temperature, and deuterium concentration. BCA II has 27 positively charged residues: 18 lysine and 9 arginine residues. There are 30 negatively charged residues: 19 aspartate and 11 glutamate residues. The side chains of all 18 lysine residues are solvent exposed.[2] The N-terminal serine residue is acetylated (when isolated from bovine erythrocytes). Each rung of the charge ladder is probably composed of an approximately statistical mixture of regioisomers.[49] Bringing about changes in net charge of ~ 16 units with conventional methods such as site-directed mutagenesis or changes in pH would require multiple rounds of mutagenesis or changes of several units in pH.

Experimental Design

BCA II Charge Ladders. Lyophilized bovine carbonic anhydrase II (E.C. 4.2.1.1) was purchased from Sigma, and resuspended in 100-mM HEPBS buffer (pH 9.0) for reaction with acetic anhydride or butyric anhydride. Charge ladders of BCA II were

produced by allowing BCA II to react with different amounts of acetic anhydride as previously described.[5] Protein charge ladders were repeatedly concentrated and were diluted in 10 mM phosphate (pH 7.4) using a Centricon centrifugal filtration device (10,000 MW; Millipore) in order to remove HEPBS buffer and acetic acid. Aliquots of acetylated BCA II (80 μM; 10 mM phosphate, pH 7.4) were flash frozen with N_2 (l) for analysis with ESI-MS, capillary electrophoresis (CE) and differential scanning calorimetry (DSC). The degree of lysine acetylation was determined with ESI-MS and CE. The perbutyrated derivative of BCA II was produced and characterized using the same procedure as the peracetylated protein, with the exception of the duration of reaction. Butyric anhydride is less soluble in water than acetic anhydride, and the reaction (an emulsion) was allowed to proceed for 2 days at 4 ºC.

Measuring Hydrogen-Deuterium Exchange of Protein Charge Ladders with Mass Spectrometry. H/D exchange was measured with mass spectrometry as previously described [16] with minor modifications that are described in the supplemental material.

Distinguishing Backbone and Lys-ε-NHCOCH$_3$ Amides in CAII with Multi-dimensional NMR. One difficulty that arises from using a Lys-NH_3^+ protein charge ladder to study the exchange of amide hydrogen in proteins is that the acetylation of lysine-ε-NH_3^+ generates an additional amide hydrogen on the lysine side chain (i.e., lys-ε-NH_3^+ + $(CH_3CO)_2O$ → lys-ε-**NH**$COCH_3$ + CH_3COOH +H^+). The mass spectrometric tools that we use to measure H/D exchange can not distinguish the amide hydrogen on an acetylated side chain from amide hydrogen on the backbone. We have, therefore, also used multi-dimensional NMR (which can distinguish side chain and backbone) to measure the rate of amide hydrogen exchange specifically at the acetyl side chains of acetylated lysine residues.

The NMR experiments were carried out on Bruker 600 MHz and 750 MHz spectrometers equipped with cryoprobes. Sensitivity-enhanced TROSY version[50] of the HSQC experiments were used to record HSQC spectra.[51] For D_2O exchange experiments, a concentrated sample of HCA II (3.5 mM) in water was diluted 10 fold in deuterated buffer (pD 7.4, 10 mM phosphate). In order to obtain a "zero-time point", an aliquot of

the same concentrated sample was diluted 10 fold in buffered H_2O (pH 7.4, 10 mM phosphate). A control spectrum in water was recorded under identical conditions. A detailed description of the experimental parameters of NMR experiments is included in the Supplemental section.

The H/D exchange of lys-ε-$NHCOCH_3$ in CA II was exclusively measured by recording the first HN plane in an HNCO experiment.[52] This two dimensional experiment (referred to as 2D-HN-HNCO) is the first plane of the HNCO experiment, in which there is no evolution of the carbonyl frequency. This 2D experiment will exclusively detect the Nitrogen-Proton correlation of those amides that are directly attached to a ^{13}C-enriched carbonyl group. This selection in the H-N plane relies on the preparation of isotopically enriched CAII in which only the amides of the lysine side chain are attached to a ^{13}C carbonyl. This selective enrichment is achieved by growing cells in ^{12}C glucose media and acetylating the purified protein with acetic anhydride that is ^{13}C enriched only at the carbonyl position. A TROSY version of the HNCO experiment where the nitrogen dimension is incremented in a semi-constant time fashion was employed to collect 2D-HN-HNCO planes. Hydrogen-deuterium exchange experiments were carried out as described above, where each H-N plane was recorded in 15 min. The data were processed with NMRPipe and the intensities of the peaks were measured using the program Sparky.[53]

Recombinant Expression and Purification of ^{15}N–labeled Carbonic Anhydrase II. Human carbonic anhydrase II (HCA II) was recombinantly expressed in *E. coli* and purified as previously described.[54] *E. coli* cells expressing HCA II were grown in M9 minimal media enriched with $^{15}NH_4Cl$ in ~90% D_2O. The cells were grown to an OD_{600} of 0.7 at 37 °C and induced for 10-12 hrs at 30 ºC with 1.5 mM IPTG. Zinc Chloride ($ZnCl_2$, 200-μM) was added before induction. The cells were lysed by sonication and centrifuged. HCA II was purified as previously described.[54] In order to remove deuterium from labile sites, solutions of purified protein were heated in phosphate-buffered H_2O (10 mM, pH 8.4) at 35 ºC for 2 days. Proteins were then transferred to 10 mM phosphate buffer (pH 7.0) with centrifugal filtration devices and stored at 4 °C for H/D exchange experiments.

Measuring the Effects of Lysine Acetylation on the Rate of Backbone Amide H/D Exchange in Model Amino Acids. In order to determine how neutralizing the ε-NH_3^+ of lysine by acetylation affected the rate of H/D exchange of the *backbone* amide of lysine, we used NMR spectroscopy to compare the H/D exchange of N-α-acetyl-L-lysine-N-methylamide (abbreviated: Ac-Lys(ε-NH_3^+)-NHMe) and the analogous ε-$NHCOCH_3$ derivative (abbreviated: Ac-Lys(ε-$NHCOCH_3$)-NHMe). These derivatives of lysine are 'models' of lysine in polypeptides in that the α-NH_3^+ group has been acetylated (yielding a "backbone" amide; the ε-NH_3^+ group is also acetylated in the ε-$NHCOCH_3$ derivative yielding a side-chain amide). The α-COO^- group has also been converted into a $CONHCH_3$ (yielding a second "backbone" amide). The rate of exchange of each amide was measured at pD 4.5, 5 °C, 20 mM acetate (the rate of exchange is too fast at neutral pH to be measured using our methods). In order to resolve the "side-chain" amide groups from "backbone" amide groups, we acquired NMR spectra on a 900 MHz spectrometer (Bruker). Nuclear Overhauser effect spectroscopy (NOESY) and Total Correlation Spectroscopy (TOCSY) were performed in order to assign NMR signals unambiguously to particular amides in two relevant structures: Ac-Lys(ε-NH_3^+)-NHMe and Ac-Lys(ε-$NHCOCH_3$)-NHMe. Additional experimental details can be found in the supporting information.

Differential Scanning Calorimetry (DSC). To determine the effect of lysine acetylation on the conformational stability of BCA II, partial charge ladders were analyzed by differential scanning calorimetry (DSC). DSC was carried out on a VP-DSC instrument (MicroCal) with a scan rate of 1 ºC/min. Protein samples (~25 μM; pH 7.4, 10 mM phosphate) were degassed prior to analysis. Raw DSC data was smoothed and deconvoluted using Origin 5.0 (MicroCal).

Capillary Electrophoresis (CE). The change in surface charge for each rung of the charge ladder was confirmed by capillary electrophoresis (CE). Capillary electrophoresis was performed as previously described using a Beckman PACE instrument.[55]

Results and Discussion

Preparation and Characterization of Charge Ladders of Bovine Carbonic Anhydrase II. Proteins with different degrees of acetylation (as measured by ESI-MS and capillary electrophoresis, CE) were prepared by causing BCA II to react with different molar equivalents of acetic anhydride. For example, a solution of proteins with 4-8 acetylations was prepared by reaction with eight molar equivalents of acetic anhydride (Figure 1); the most abundant species had ~ 6 modifications (according to mass spectrometry and capillary electrophoresis, Figure 1A-C). This partial charge ladder was denoted "BCA-Ac(~6)". The relative abundance of each rung is similar when analyzed by either mass spectrometry or capillary electrophoresis (during CE, proteins are detected by their absorbance at 214 nm). This similarity in abundance when solutions were measured by CE and ESI-MS demonstrated that each rung had a similar ionization efficiency during electrospray ionization (Figure 1D).

We analyzed the partial charge ladders denoted "BCA-Ac(~6)", "BCA-Ac(~9)" and the peracetylated protein (with 18 lysine modifications; denoted BCA-Ac(18)) using differential scanning calorimetry (DSC), in order to determine how the acetylation of lysine affected the thermostability of folded BCA II. Combining various partial ladders with unmodified and peracetylated BCA II resulted in a full charge ladder with 19 rungs: 18 variously acetylated derivatives and the unmodified protein (Figure 1E,F).

Higher (More Highly Charged) Rungs of the BCA II Charge Ladder have Lower Conformational Stability. The thermal denaturation of unmodified BCA II (denoted BCA-Ac(0)), peracetylated BCA II (BCA-Ac(18)), and partial BCA II charge ladders produced well-defined changes in heat capacity. The endothermic transitions shown in Figure 2A were generated by deconvoluting the raw data (using Origin 7.0). Integration of each endotherm yielded temperatures of the melting transition (T_m). For BCA II-Ac(0), T_m = 69.3 ºC; for BCA-Ac(~6), T_m = 65.7 ºC; for BCA-Ac(~9), T_m = 63.9 ºC; for BCA-Ac(18), T_m = 49.8 ºC. We observed a non-linear relationship between the number of acetyl modifications to BCA II and its thermostability; the first ~9

modifications lower the T_m by 5.4 ºC; the next 9 modifications, however, lower the T_m by 14.1 ºC (Figure 2B).

Measuring Amide H/D Exchange in a BCA II Charge Ladder with Electrospray Ionization-Mass Spectrometry (ESI-MS). The mass spectrometric method that we use to measure H/D exchange (illustrated in Figure 3) will measure the *global* exchange of hydrogen in BCA II, and can not distinguish individual residues.[56] We expressed the kinetics of H/D exchange for each rung of the charge ladder in terms of its number of *unexchanged* hydrogens (as opposed to the number of *exchanged* hydrogen or incorporated deuterons) because the number of *unexchanged* hydrogens provides a more accurate description of the overall structure of a protein than does the number of exchanged hydrogens or incorporated deuterium.[57] The number of unexchanged hydrogens (denoted H_{unex} in Equation 4) for each rung is calculated by subtracting the measured mass of each rung throughout the H/D exchange experiment (denoted $M[D]_{native}$; illustrated in Figure 3B) from the measured mass of each perdeuterated rung (denoted $M[D]_{unfolded}$; Figure 3C).

$$H_{unex} = M[D]_{unfolded} - M[D]_{native} \qquad (4)$$

Hydrogen/deuterium exchange is initiated and measured as previously described.[58] Briefly, a concentrated solution of protein charge ladder was diluted ten-fold from buffered H_2O (20 mg/mL protein, 15 °C, 10 mM PO_4^{3-}, pH 7.4) into buffered D_2O (15 °C, 10 mM PO_4^{3-}, pD 7.4; Figure 3A; see supplemental information for additional experimental details). Aliquots were removed over time, and isotopic exchange was immediately quenched by diluting aliquots again (1:10) into ice-chilled, acidic, aqueous buffer (0 ºC, 100 mM PO_4^{3-}, pH 2.4). Solutions were then injected onto a short HPLC column (in order to remove salts that suppress ESI) that was chilled on ice and coupled to the ESI-MS (Figure 3B). During quenching and analysis with LC-ESI-MS, deuterons on side chain functionalities such as carboxylic acid, indole, guanidinium or alcohol groups will typically undergo back-exchange with water.[59, 60] The LC-ESI-MS methods we use will therefore measure the exchange of hydrogen primarily at the amide nitrogen, and not

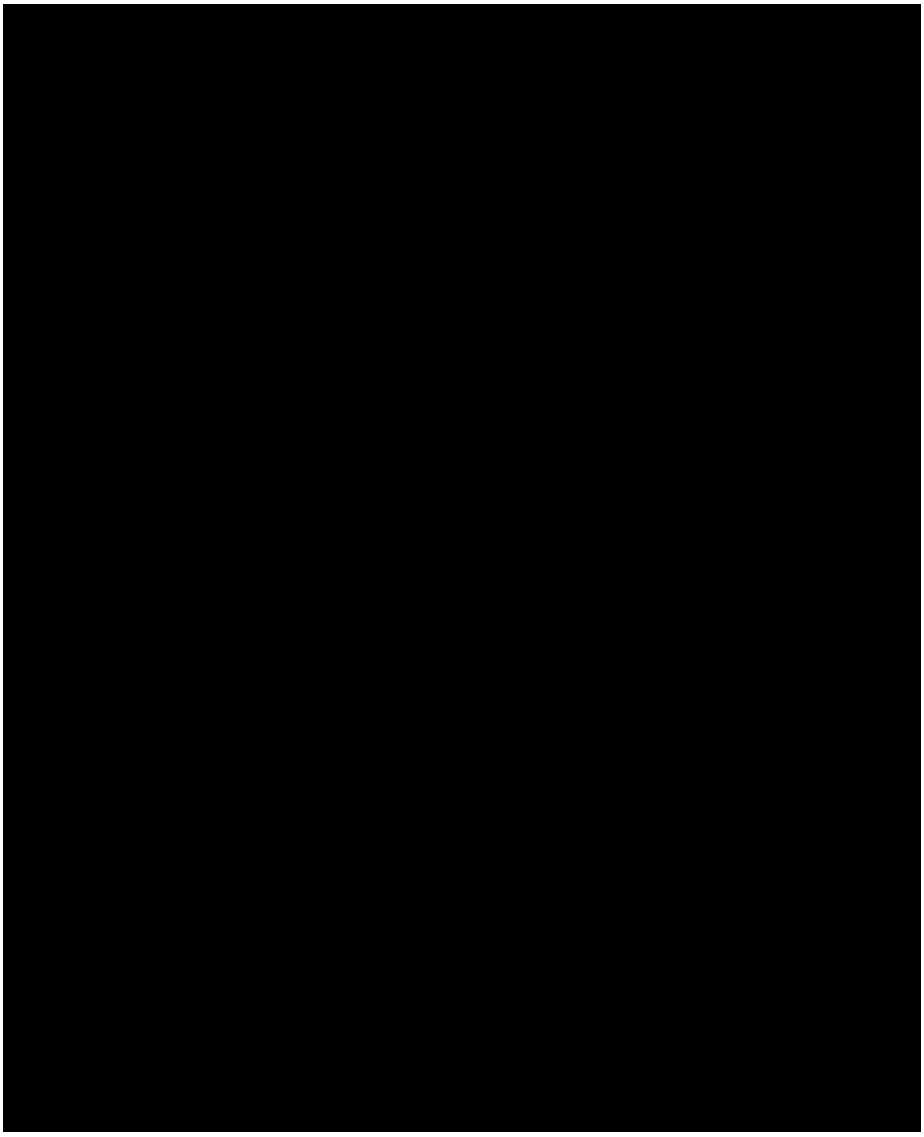

Figure 1. Electrospray ionization mass spectrometry (ESI-MS) of a lys-ε-NHCOCH$_3$ charge ladder of BCA II. A) Rungs of the lys-ε-NHCOCH$_3$ charge ladder are well resolved with electrospray ionization mass spectrometry (ESI-MS). The +35 molecular ion (A) and the mass reconstruct (B) are shown for a partial charge ladder (in H_2O) having between 2 and 11 acetylated lysines (the most abundant rung is Ac 6). C) Capillary electrophoresis of the same sample shows a similar distribution of acetylated proteins (between 2 and 11 modifications; the most abundant species had 6 modifications). D) Integrated values of intensity (from the mass spectra in B) for Ac(1)-Ac(11) plotted against integrated values of absorbance (from the electropherogram in C).

The approximately linear correlation demonstrates that each rung of the charge ladder has a similar ionization efficiency during ESI-MS (although the higher rungs have lower relative values of absorbance due to their greater mobility during CE). E) The mass spectra of a full protein charge ladder was prepared by mixing partial charge ladders with unmodified and peracetylated BCA II. The charge state distribution of BCA II is shifted to higher m/z values (i.e. lower positive charge states) as the degree of acetylation increases. The predominant charge states are +34 to +37 for unmodified BCA II and +30 to +28 for peracetylated BCA II. F) Mass reconstruct of spectra of full charge ladder showing all 19 rungs (in D_2O).

hydrogen on rapidly exchanging groups.[59, 61] A substantial number of amide deuterons, however, will also undergo "back-exchange" with solvent during quenching and LC-ESI-MS (Figure 3B). Consequently, the number of deuterons that are incorporated into BCA II during the in-exchange experiment (Figure 3A) will be underestimated unless this back-exchange is taken into account. The percent of deuterons that undergo back-exchange (% BE) is calculated as the difference between the *measured* mass of each perdeuterated rung and the *theoretical* mass of each perdeuterated rung; the perdeuterated ladder is prepared by thermally unfolding the proteins (Figure 3C). We found that approximately 27 % of amide deuterons had undergone back-exchange with solvent during quenching and analysis with LC-ESI-MS (Supplemental Table 1). This value is consistent with reported values that involved similar LC-ESI-MS methods.[58]

We emphasize that the acetylation of lysine results in an additional amide (lys-ε-NH_3^+ + $(CH_3CO)_2O$ → lys-ε-**NH**$COCH_3$ + CH_3COOH +H^+). Consequently, we expect the maximum number of deuterons that can be incorporated into amide sites of *unfolded* BCA II to increase (in 90 % D_2O) by approximately 0.9 with each additional modification. This result is in fact what we observed by thermally unfolding the charge ladder in deuterated buffer and measuring the mass of each rung (Supplemental Table 1). The number of deuterons incorporated into unfolded BCA II increased by 0.5-1.0 with each higher rung of the ladder (Supplemental Table 1). The exchange of the amide hydrogen of lys-ε-$NHCOCH_3$ can, therefore, be measured with our protocol and apparatus, and these hydrogens are thus included when calculating the number of unexchanged hydrogens for each rung.

H/D Exchange of the BCA II Charge Ladder. Figure 4A shows the kinetics of H/D exchange of the charge ladder monitored by mass spectrometry. In the case of the first rung, BCA-Ac(0), there are 37 hydrogen atoms that exchange with solvent before the first time point (typically ~20 s). These hydrogens exchange rapidly with solvent because, presumably, they are not buried away from solvent and/or not located in a highly structured region. Approximately 85 hydrogens in BCA-Ac(0) remain unexchanged with solvent after 100 minutes (90 % D_2O, pD 7.4, 15 ºC). These 85 hydrogen exchange slowly because, we assume, they are hydrogen bonded and/or are

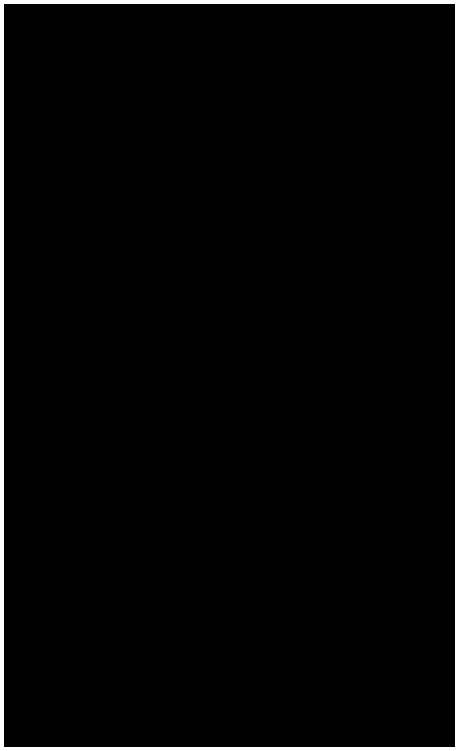

Figure 2. Lysine acetylation decreases the thermostability of BCA II. A) Thermal denaturation of unacetylated BCA II (denoted Ac(0)), partially acetylated BCA II (Ac(~6) and Ac(~9)) and peracetylated BCA II (Ac(18)) measured by differential scanning calorimetry (DSC). Integration of peaks produced melting temperature (T_m) values of 69.3 °C (Ac(0)), 65.7 °C (Ac(~6)), 63.9 °C (Ac(~9)), and 49.8 °C (Ac(18)). B) Plot of T_m of Ac(0), Ac(~6), Ac(~9), Ac(18) versus the average number of modifications.

Figure 3. Measuring the amide hydrogen-deuterium exchange of proteins using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). A) H/D exchange was initiated by diluting concentrated protein solutions (1:10 v/v) from buffered H_2O into buffered D_2O. B) The mass of the protein was measured as a function of time by quenching the isotopic exchange of an aliquot with low pH buffer (pH 2.4, 100 mM PO_4^{3-}) and injection onto an LC-ESI-MS apparatus that was equilibrated at 0 ºC (the ionization solvent used in LC-ESI-MS is 0.3 % formic acid, 49.85 % acetonitrile and 49.85 % H_2O). We refer to this measured mass as $M[D]_{native}$. C) The perdeuterated protein is prepared by thermally unfolding an aliquot from B and measuring the mass as shown in C. We refer to this measured mass as $M[D]_{unfolded}$. Deuterons on side chain functionalities (-OH, -COOH, $-NH_3^+$, $-C(NH_2)_2^+$) rapidly exchanged with H_2O during the analysis with HPLC-MS (e.g., during steps "B" and "D"). The number of unexchanged amide hydrogen (H_{unex}) at any given time during the experiment is calculated as $H_{unex} = M[D]_{unfolded} - M[D]_{native}$.

buried from solvent (according to the *solvent penetration* or *local unfolding* models of H/D exchange).

For visual clarity, Figure 4A shows the kinetic profile for only seven rungs of the charge ladder (e.g., BCA-Ac(0), (3), (6), (9), (12), (15) and (18); data for all 19 rungs are included in supporting information). Figure 4A shows that the number of unexchanged hydrogens (after 100 min, pD 7.4, 15 ºC) in the charge ladder increases from ~85, for the first rung (BCA-Ac(0)), by approximately one hydrogen for each additional rung. For example, the first rung has ~ 85 unexchanged hydrogens and each additional rung (N), has approximately 85 + N unexchanged hydrogens. The sixteenth rung (BCA-Ac(15)) thus has 101 unexchanged hydrogens (Figure 4A, see also Supplemental Table 1).

The number of unexchanged hydrogens present after 80 min (pD 7.4, 15 ºC) in each rung is plotted in Figure 4B as a function of the number of acetylated lysine residues. We chose to plot the data points at 80 minute because the change in mass began to plateau at 80 min; the mass values at 80 min have, therefore, lower variation than values at, for example, 120 s. The slope of this plot (Figure 4B) is 0.96 ± 0.05 $H_{unex} \cdot$ Lys-$NHCOCH_3^{-1}$; this slope demonstrates that each acetylation results in approximately 1 hydrogen that is protected from solvent exchange after 80 min in D_2O. The increase of approximately one H_{unex} with each rung of the ladder is not observed at the highest rungs: the last four rungs of the charge ladder have nearly equal numbers of unexchanged hydrogens after 80 min. It is important to remember that each rung of the charge ladder represents, to extents that depend on the extent of acetylation, a mixture of regioisomers. The kinetics of H/D exchange measured for each rung (Figure 4A, Supplemental Table 1 and 2) are, therefore, a population-weighted average value that represents the H/D exchange of all regioisomers within the rung. We note that the mass distribution of each rung of the charge ladder (Figure 4C) remained unimodal and shifted to higher and higher values of mass as a result of deuteration—as opposed to being bimodal, with a lower mass peak (protonated protein) decreasing in intensity and a higher mass peak (deuterated protein) increasing in intensity. The unimodal distribution that we observed suggests that the exchange of most hydrogen of each rung occurs by a predominantly EX2 mechanism. Further support for an EX2 mechanism of exchange for the slowest exchanging hydrogen in both acetylated and unmodified BCA II is shown in Supplemental Figure 1B: the

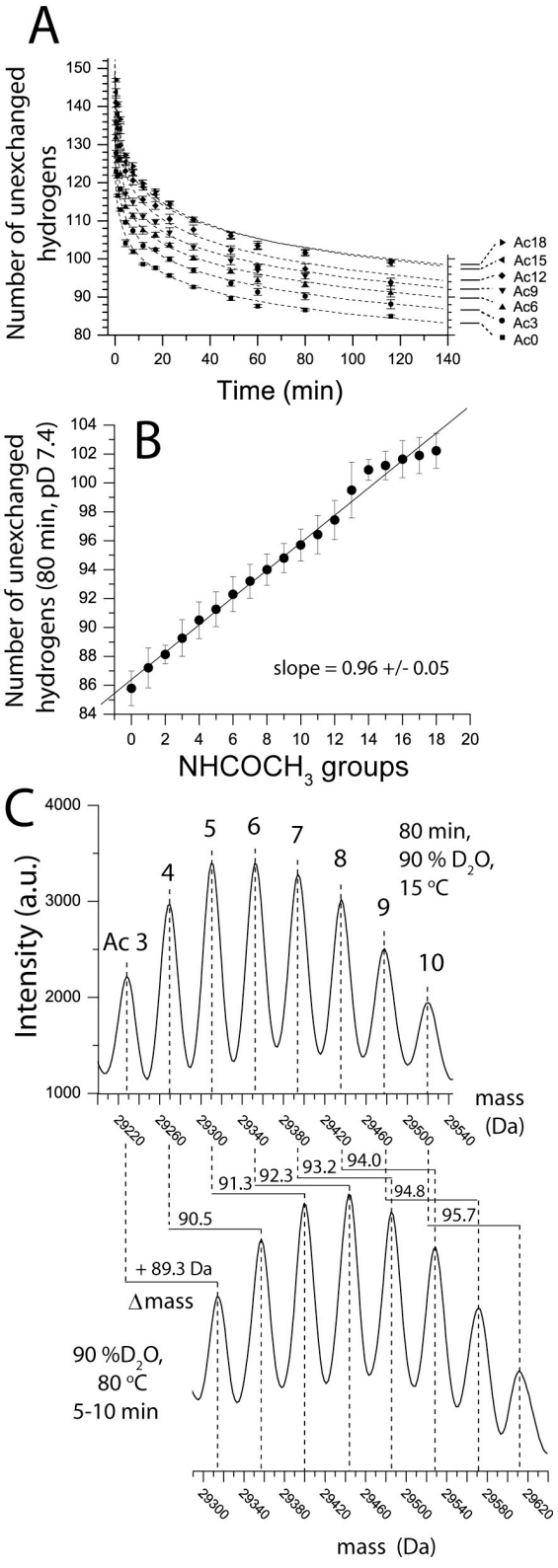
A
Number of unexchanged hydrogens
Time (min)
Ac18
Ac15
Ac12
Ac9
Ac6
Ac3
Ac0
B
Number of unexchanged hydrogens (80 min, pD 7.4)
slope = 0.96 +/- 0.05
NHCOCH3 groups
C
Intensity (a.u.)
Ac 3
4
5
6
7
8
9
10
80 min,
90 % D2O,
15 °C
mass (Da)
91.3
92.3
93.2
94.0
94.8
95.7
90.5
+ 89.3 Da
Δmass
90 %D2O,
80 °C
5-10 min
mass (Da)

Figure 4. Lysine acetylation decreases the rate of H/D exchange of BCA II as measured by ESI-MS. A) H/D exchange kinetics of the BCA II charge ladder (90 % D_2O, pD 7.4, 15 °C). For visual clarity only BCA-Ac(0), (3), (6), (9), (12), (15) and (18) are shown. BCA-Ac(0) retained ~85 unexchanged hydrogens after 100 minutes in D_2O. The higher rungs are more protected from H/D exchange. BCA-Ac(3) retained ~ 87 unexchanged hydrogens after 100 minutes; BCA-Ac(6), 90; BCA-Ac(9), 93 and BCA-Ac(12), 97. The last four rungs, BCA-Ac(15) through BCA-Ac(18) have nearly superimposable exchange profiles and retain ~ 100 unexchanged hydrogens after 100 min. Error bars represent the standard deviation of average mass values calculated from seven charge states for each rung. B) A plot of the number of unexchanged hydrogens in BCA II (after 80 min in D_2O, 15 ºC, pD 7.4) with respect to the number of lys-$NHCOCH_3$. Error bars represent the standard deviation from three separate experiments. A linear fit of the entire data set yielded a slope of 0.96 ± 0.05 $H_{unex} \cdot$ Lys-$NHCOCH_3^{-1}$. C) Mass reconstruct showing BCA-Ac(3) through BCA-Ac(10) after 80 minutes in 90% D_2O, pD 7.4, 15 ºC (top) and after the same sample is heated and the proteins are unfolded. Each higher rung incorporates approximately one additional deuteron upon thermal unfolding as observed by an increase of 0.8-1.0 Da in the mass for each rung.

number of unexchanged hydrogen after > 5 min for both peracetylated BCA II and unmodified BCA II is similarily dependent upon the pD of solvent.

The kinetic plots in Figure 4 were fit with triexponential functions in order to extract kinetic parameters for "fast," "medium" and "slow" exchanging hydrogens, as previously described.[58] This analysis is described in the supplementary material and the results for all 19 rungs of the charge ladder are listed in Supplemental Table 2. From this kinetic analysis we can estimate the reduction in the rate of H/D exchange that occurs from acetylation, if we assume (for the moment—the matter is solved experimentally below; see Figures 5 and 6) that the additionally protected hydrogens are located on the backbone of BCA II and not on the side chain of acetylated lysine residues and if we assume that the hydrogen that become protected by acetylation were *not* protected from exchange before acetylation. From our kinetic analysis of the plots in Figure 4A and Supplemental Figure 1 we conclude that unmodified BCA II has approximately 36.2 ± 3.6 amide hydrogens that exchange with a rate constant $k = 5.8 \pm 3.6 \cdot min^{-1}$; there are 24.3 ± 2.9 amide hydrogens that exchange more slowly with a rate constant $k = 1.9 \pm 0.5 \cdot 10^{-1}\ min^{-1}$; 96.4 ± 2.7 amide hydrogens are the slowest to exchange with a rate constant $k = 1.9 \pm 0.5 \cdot 10^{-3}\ min^{-1}$. For peracetylated BCA II (the kinetic results of the other 17 rungs are listed in Supplemental Table 2), 34.8 ± 3.1 hydrogens exchange with a rate constant $k = 4.3 \pm 1.1 \cdot min^{-1}$; 29.1 ± 2.9 hydrogens with a rate constant $k = 0.9 \pm 0.2 \cdot 10^{-1}\ min^{-1}$; and 112.5 ± 3.6 hydrogens with a rate constant $k = 1.1 \pm 0.3 \cdot 10^{-3}\ min^{-1}$. The additionally protected hydrogens in peracetylated BCA II exchange, therefore, with a rate constant $k = 1.1 \pm 0.3 \cdot 10^{-3}\ min^{-1}$. We do not know the rate constant at which these hydrogens underwent exchange prior to acetylation, but if we assume that the rate constant is between $5.8 \pm 3.6 \cdot min^{-1}$ and $1.9 \pm 0.5 \cdot 10^{-1}\ min^{-1}$ (e.g., the rate constants for "fast" and "medium" exchanging hydrogens in unmodified BCA II) then we can make a zeroth order approximation that each acetylation reduces the rate of H/D exchange of amides hydrogens in BCA II by at *least* two or three orders of magnitude.

Is the Additional Protected Hydrogen in Each Rung Located on the Protein Backbone or Side Chain of Lys-ε-NHCOCH$_3$? The simplest explanation (numerically, but not necessarily chemically) for the effects of acetylation on the rate of H/D exchange

within the charge ladder—that is, the ~1:1 relationship between the number of modifications and the increased number of unexchanged hydrogens—is that the additional hydrogen that were protected in each rung are those amides introduced onto the side chain of lysine via acetylation. All 18 lysine in BCA II are, however, located on the surface of the protein and X-ray crystallography of peracetylated BCA II revealed that the lys-ε-$NHCOCH_3$ groups are neither buried nor inaccessible to solvent.[8] A few of these amide groups were engaged in intramolecular hydrogen bonds, for example, with aspartate-β-COO^- functionalities.[8] Nevertheless, the rate of exchange of lys-ε-$NHCOCH_3$ are difficult to predict and not necessarily slower than backbone –CONH hydrogen.

In order to determine if the additional hydrogens that were protected in the charge ladder were those amide hydrogen on lys-ε-$NHCOCH_3$ (and not backbone amide hydrogen), we measured the rate of H/D exchange of lys-ε-$^{15}NH^{13}COCH_3$ on peracetylated CA II using multidimensional NMR. For these experiments, we recombinantly expressed, and purified, carbonic anhydrase II proteins that were labeled with ^{15}N and ^{12}C, and then acetylated these proteins, after purification, with ^{13}C-labelled acetic anhydride ($(CH_3{}^{13}CO)_2O$). We used the human variant of carbonic anhydrase II (HCA II) for NMR experiments because an expression system was readily available. The human variant has 24 lysines and, when expressed in the prokaryotic expression system, is not acetylated at its N-terminus (there are, therefore, 25 R-NH_3^+ rather than 18 for BCA II). HCA II is similar to BCA II in its sequence and structure [62, 63] (e.g., the two proteins have 79 % sequence homology); the net negative charge of HCA II at pH 8.4 was measured with CE to be –2.3; the net charge of BCA II was measured to be –3.3 under similar conditions. [49]

Using a TROSY (Transverse Relaxation Optimized Spectroscopy) version of the HSQC (Heteronuclear Single Quantum Coherence) NMR experiment we have demonstrated that amide hydrogens on lys-ε-$NHCOCH_3$ exchange rapidly with solvent ($t_{1/2} << 30$ min, 15 °C, pD 7.4) and are not the hydrogen that became protected as a result of acetylation (Figure 5). This result demonstrates that the additional hydrogen that were protected in each rung of the charge ladder are main-chain amides located somewhere on the backbone of the protein (Figure 5). An explanation of the results of NMR experiments that led us to this conclusion is as follows.

An overlay of the HSQC NMR spectra of HCA II (blue) and peracetylated HCA II (red) is shown in Figure 5A. A HSQC experiment correlates the amide nitrogen with the amide hydrogen; a cross peak is observed for each amide N-H pair. Each peak, therefore, represents an amide N-H that is in a unique chemical environment. Other N-H signals (i.e., δ-guanidino, β- and γ-$CONH_2$, ε-NH_3^+, δ-NH, β-imidazole and β-indole) are typically not observed because they exchange too rapidly to be detected, or because they are suppressed in these types of experiments.[64] Many peaks in the HSQC spectra of HCA II and peracetylated HCA II do not overlap (this difference is not surprising, considering that the chemical environment of many residues will be altered by the neutralization of lys-ε-NH_3^+). The majority of peaks in the spectra of both proteins were dispersed and well resolved, indicating that both proteins were folded.[64, 65]

The HSQC spectrum of HCA II included approximately 260 observable peaks; this indicates that approximately 260 amide N-H species in HCA II were in unique chemical environments. This number is approximately equal to the number of amides in the HCA II polypeptide: HCA II has 259 residues; 17 are proline residues (which do not have backbone NH groups and are not observable in this type of HSQC spectrum). There are, therefore, 242 backbone amides in HCA II. This difference of 18 peaks is plausibly due to residues that exist in more than one conformation. The HSQC spectrum of peracetylated HCA II contained 310 peaks (Figure 5A, red spectrum). The additional 50 peaks in the spectrum of peracetylated HCA II are due, in part, to the 25 additional amides introduced by acetylation. The additional ~ 25 peaks, beyond those that can be attributed to acetylation, are likely to represent amides in different HCA II conformers (these could be amides located on the backbone of HCA II, or the lysine-ε-$NHCOCH_3$ or both).

The HSQC spectrum of peracetylated HCA II shows a group of peaks (at 7-8 ppm, 126-129 ppm; Figure 5A) that includes 22 resolved peaks and a broad set of overlapping peaks that appear as a cluster of approximately 6 peaks. This entire group is conspicuous because it is not present in the HSQC spectrum of unmodified HCA II. In order to determine if these amides were coupled to ^{13}C and therefore represented the side chain amides of lysine-ε-$NHCOCH_3$ (and possibly the acetylated N-terminus), we performed a TROSY version of an HNCO NMR experiment. An HNCO experiment is a

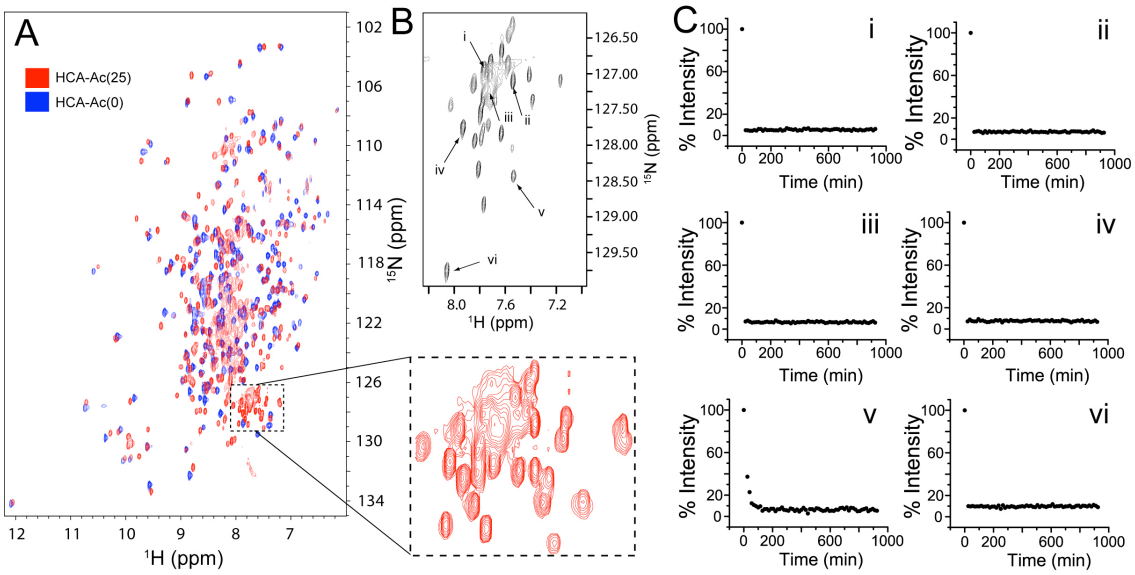

Figure 5. Hydrogen-deuterium exchange of peracetylated HCA II measured by multidimensional NMR. A) Overlay of HSQC ^{1}H-^{15}N NMR spectra for HCA II (blue) and peracetylated HCA II (red; denoted HCA-Ac(25)). HCA II was acetylated with ^{13}C-labelled acetic anhydride ($(CH_3{}^{13}CO)_2O$). HCA II was expressed by *E. coli* in media enriched with ^{15}N, ^{12}C and ^{2}H. In order to remove deuterons from non-alkyl groups, HCA II proteins were heated at 35 °C in H_2O for 48 hours prior to analysis with NMR. The dashed box highlights, for HCA-Ac(25), a set of 22 resolved peaks and a broad set of overlapping peaks (containing 4-6 peaks) that are not observed in HCA-Ac(0). B) The 2D-HN-HNCO of a TROSY-HNCO experiment; each signal represents a correlated ^{1}H-^{15}N that is also correlated to ^{13}C=O. C) Plots of the intensity of signals from the TROSY-HNCO(2D-HN-HNCO) experiment as a function of time (in deuterated buffer; 15 ºC, pD 7.4, 10 mM PO_4^{3-}). The H/D exchange of only six different amides (i-vi) are shown here (the remaining are shown in Supplemental Figure 3).

triple resonance NMR experiment that correlates the nuclear spin of the ^{1}H and ^{15}N of the amide to the ^{13}C=O that is attached directly to the nitrogen. The H-N plane of an HNCO experiment will, therefore, reveal the side chain N-H groups of lys-ε-$^{15}NH^{13}COCH_3$ (because the protein was expressed in a media containing ^{12}C Glucose and acetylated with $(CH_3{}^{13}CO)_2O$. The triple resonance spectrum shows that each N-H signal in this conspicuous group is coupled to ^{13}C and therefore represents the N-H from lysine-ε-$NHCOCH_3$ (Figure 5B). Twenty-two resolved peaks were observed in the triple resonance spectrum in addition to a broad set of overlapping peaks (Figure 5B). No other signals were observed in the N-H plane of this triple resonance spectrum (Supplemental Figure 2).

The rate of H/D exchange of the acetyl amides was measured by monitoring the disappearance of each peak in D_2O. Concentrated solutions of peracetylated HCA II (3.5 mM) were diluted 1:10 (v/v) into deuterated buffer (pD 7.4, 15 ºC, 10 mM PO_4^{3-}). An aliquot from the same concentrated solution was diluted 1:10 into buffered H_2O (pH 7.4, 15 ºC, 10 mM PO_4^{3-}) in order to obtain a "zero" time point. The decrease in intensity of 6 of the peaks are shown with respect to time in D_2O (Figure 5C). Plots of the remaining 22 peaks are shown in Supplemental Figure 3. Twenty one of the peaks representing amides from lysine-ε-$NHCOCH_3$ (and α-$NHCOCH_3$) disappeared before the first time point could be measured (e.g., in less than 27 minutes: 12 min was required to dilute the concentrated HCA II protein into deuterated buffer, shim the magnet, tune the probe, and begin scanning; each spectrum is a collection of scans collected over 15 minutes).

In order to illustrate the persistence of an NMR signal that represents a backbone amide in peracetylated HCA II that *is* protected from H/D exchange, we chose three N-H systems (that are not correlated with ^{13}C and are presumably backbone amides) from the HSQC spectrum of peracetylated HCA II. The intensity of each of these peaks is plotted as a function of time in D_2O (Supplemental Figure 4). These three N-H systems exchange at different rates (and were chosen based on their varied rate of exchange).

Together, the data presented in Figure 5 demonstrates that the amides from lysine-ε-$NHCOCH_3$ exchange too rapidly to be those that are protected within the protein charge

ladder. The hydrogens that are becoming protected from exchange as a result of acetylation are, therefore, located on the backbone of BCA II.

Acetylating the ε-NH_3^+ Group of Model Lysine Decreases the Rate of H/D Exchange of its Backbone Amide. In order to determine how the neutralization of ε-NH_3^+ affects the rate of H/D exchange at the backbone amide of, specifically, lysine we measured the rate of exchange of a model of lysine (N-α-acetyl-L-lysine-N-methylamide; abbreviated: Ac-Lys(ε-NH_3^+)-NHMe) as well as the ε-$NHCOCH_3$ derivative (abbreviated: Ac-Lys(ε-$NHCOCH_3$)-NHMe) with ^{1}H NMR spectroscopy. The rate of H/D exchange was monitored by the disappearance of signal for the α-nitrogen amide (e.g., the 'left-handed' amide (amide 2 in Figure 6), with respect to the α-carbon in Figure 6) at ~ 8.10 ppm and the "right handed" amide (amide 1 in Figure 6) which appears at ~ 7.83 ppm (Figure 6A). We found that the neutralization of ε-NH_3^+ in model lysine resulted in a significant decrease in the rate of exchange of both the right and left backbone amide (Figure 6B and 6C). The data in Figure 6 were fit to the exponential function $y = y_o + Ae^{(-x/k)}$; the time constants (k or $t_{1/2}$) for the left handed amide (denoted "1" in Figure 6) were 526 ± 6 s for Ac-Lys(ε-NH_3^+)-NHMe ($R^2 = 0.9897$) and 1639 ± 10 s for Ac-Lys(ε-$NHCOCH_3$)-NHMe ($R^2 = 0.9985$). The acetylation of the ε-NH_3^+ of model lysine, therefore, increases the half-life ($t_{1/2}$) of exchange of its backbone amide hydrogen by a factor of approximately 3.1. The neutralization of ε-NH_3^+ decreased the rate of exchange of the right handed amide (denoted "2" in Figure 6) by a lesser degree than the left handed amide. The time constants for the right handed amide were 1960 ± 22 s for Ac-Lys(ε-NH_3^+)-NHMe ($R^2 = 0.9958$) and 3278 ± 25 s for Ac-Lys(ε-$NHCOCH_3$)-NHMe ($R^2 = 0.9989$); the neutralization of ε-NH_3^+, therefore, increased the half-life of exchange by a factor of 1.7.

The decrease in the rate of exchange of the backbone amide hydrogens in model lysine that results from the acetylation of ε-NH_3^+ (e.g., a 2-3 fold decrease; Figure 6) is significantly less than the decrease that we estimate to occur in BCA II as a result of acetylation of lysine (e.g., a 100-1000 fold decrease; Figure 4, Supplemental Table 2). One plausible explanation for this disparity is that the small molecule experiments were performed at different value of pD (i.e., pD 4.5) compared to the experiments with BCA

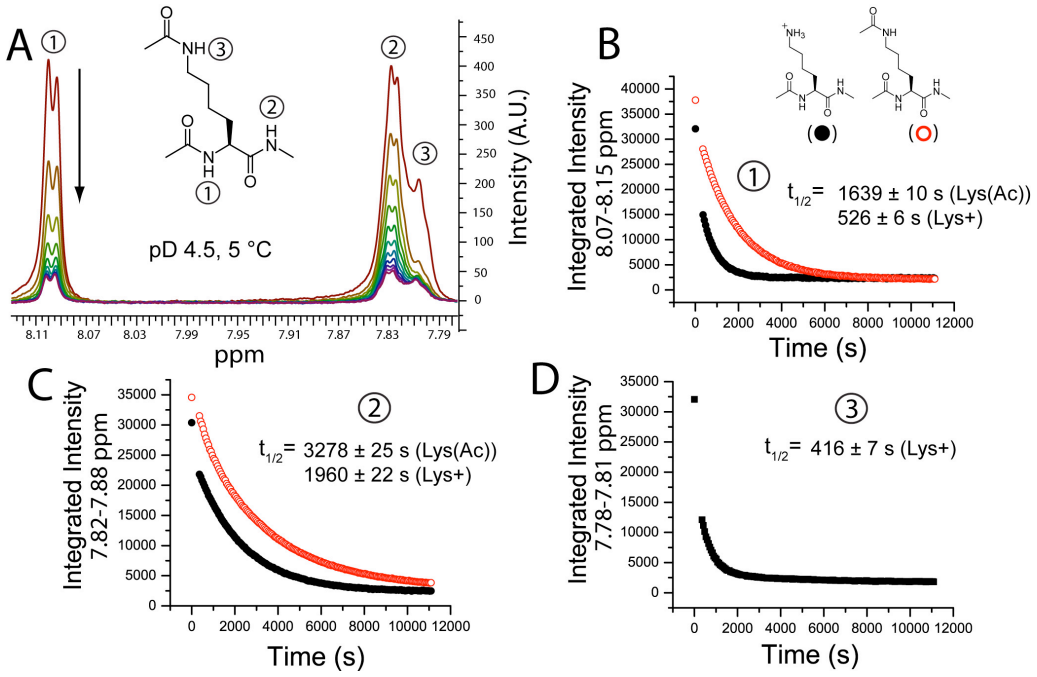

Figure 6. Neutralizing the ε-NH_3^+ group of Ac-Lys(ε-NH_3^+)-NHMe reduces the rate of H/D exchange at the backbone amide of the amino acid. The rate of backbone amide exchange for model lysine compounds Ac-Lys(ε-NH_3^+)-NHMe and Ac-Lys(ε-$NHCOCH_3$)-NHMe were measured with ^{1}H NMR spectroscopy at pD 4.5, 5°C. A) The exchange of the amide hydrogen of the α-nitrogen (e.g., the 'left-handed' amide) was monitored in both compounds by the disappearance of the signal at ~ 8.10 ppm and the 'right-handed' amide by the signal at ~ 7.83 ppm. The ε-amide hydrogen (ε-$NHCOCH_3$) of Ac-Lys(ε-$NHCOCH_3$)-NHMe appears at ~ 7.80 ppm. B-D). The amide H/D exchange of both compounds expressed as a function of the integrated signal and time. Solid black circles represent Ac-Lys(ε-NH_3^+)-NHMe and open red circles represent the neutral compound Ac-Lys(ε-$NHCOCH_3$)-NHMe. The function $y = y_o + Ae^{(-x/k)}$ was fit to each plot. B) For the left handed amide (denoted "1"), the half-life of exchange ($t_{1/2}$) = 526 ± 6 s for Ac-Lys(ε-NH_3^+)-NHMe (R^2 = 0.9897) and 1639 ± 10 s for Ac-Lys(ε-$NHCOCH_3$)-NHMe (R^2 =0.9985). The ratio of half lives = 3.1. C) The neutralization of ε-NH_3^+ decreased the rate of exchange of the right handed amide (denoted "2") by a lesser degree than the left handed amide. For Ac-Lys(ε-NH_3^+)-NHMe $t_{1/2}$ = 1960 ± 22 s (R^2 = 0.9958) and for Ac-Lys(ε-$NHCOCH_3$)-NHMe $t_{1/2}$ = 3278 ± 25 s (R^2 = 0.9989); the ratio of half

lives = 1.7. D) The ε-amide of Ac-Lys(ε-$NHCOCH_3$)-NHMe (denoted "3") exchanges faster than any of the backbone amide hydrogen: $t_{1/2}$ = 416 ± 7 s.

II (i.e., pD 7.4). Previous work has shown that model lysine similar to that studied here has a minimum rate of H/D exchange near pH 4.0.[29, 30] At pD 4.5, the exchange of amide hydrogen with deuterium is, therefore, catalyzed by D_3O^+ to a greater degree than at pD 7.4. Neutralization of ε-NH_3^+ will most likely accelerate the rate of acid (D_3O^+) catalyzed exchange (although the neutralization of ε-NH_3^+ is expected to reduce the rate of base catalyzed exchange).

We also note that the hydrogen on the side-chain amide of Ac-Lys(ε-$NHCOCH_3$)-NHMe exchanges faster than any of the backbone amide hydrogens (k = 416 ± 7 s; Figure 6D). The relatively fast rate of H/D exchange of ε–$NHCOCH_3$ compared to the two backbone amides is further support for the conclusion that the side chain amides of acetylated BCA II are not protected from H/D exchange.

Neutralization of Lysine, Charge Regulation and Hydrogen Exchange. We hypothesized that the correlation between the net negative charge of BCA II and its rate of hydrogen exchange is due—at least in part—to a manifestation of *charge regulation* at the surface of BCA II. Charge regulation occurs from the reorganization of solvent and solvent ions (i.e., H^+ or OH^-) around a functional group that undergoes a change in charge. In the case of the acetylation of lysine-ε-NH_3^+, the neutralization of the positively charged nitrogen will lower electrostatic repulsions between ε-N and H^+ and result in a decrease in the local pH (pH_{local}) relative to the pH of the bulk solvent (pH_{solv}). These differences in pH_{local} and pH_{solv} will also affect the ionization of residues that have values of pK_a within ± 3 units of pH_{solv}.[7] The lower pH_{local} at the surface of BCA-Ac(18) compared to BCA-Ac(0) would, expectedly, result in decreased rates of H/D exchange for BCA-Ac(18) compared to BCA-Ac(0). From a zeroth order approximation, however, we can not estimate quantitatively the magnitude of change to the rate of H/D exchange that would occur in BCA II as a result of a reorganization of solvent ions and adjustments in the pK_a of ionizable groups at the protein surface.

In order to begin to quantify how changes in electrostatics at the surface of BCA II (i.e., changes in local pH and the reorganization of solvent ions) that result from acetylation might affect the rate of H/D exchange, we measured the H/D exchange of unmodified and acetylated BCA II in 0.1 M and 1.0 M sodium chloride. Sodium and

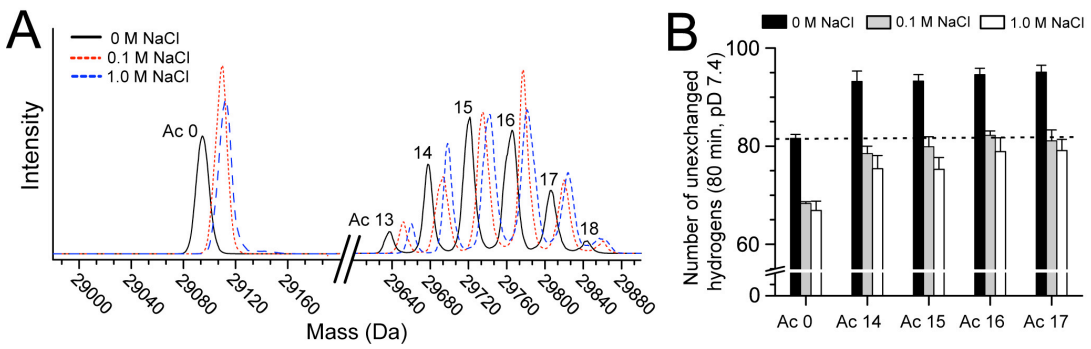

Figure 7. Sodium chloride decreases the rate of H/D exchange in both acetylated and unmodified BCA II. A) Mass spectra of unmodified BCA II and acetylated BCA II (e.g., rungs Ac(13)-Ac(18)) after 80 min in D_2O, pD 7.4, 0-1 M NaCl. The mass of each BCA II species increases with the concentration of NaCl (indicating an increase in the incorporation of deuterium). **B)** Bar graph showing the number of unexchanged hydrogens in unmodified and acetylated BCA II after 80 min in D_2O, pD 7.4, 0-1 M NaCl. The number of unexchanged hydrogens in each protein decreases with increasing sodium chloride. Unmodified BCA II in 0 M NaCl and acetylated BCA II in 1 M NaCl have a similar number of unexchanged hydrogens after 80 min in D_2O (note: the rungs corresponding to Ac(13) and Ac(18) are omitted from the bar graph because these rungs are low in intensity and did not yield precise values of mass).

chloride ions will effectively screen electrostatic interactions at the surface of the BCA II protein, for example between OH^- and lys-ε-NH_3^+. We used a partial charge ladder of BCA II for these experiments that consisted of only the higher rungs (i.e., rungs Ac(14)-Ac-(17)). Working with a partial ladder consisting of two or three abundant rungs instead of a full ladder consisting of 19 is convenient for mass spectrometric experiments that involve high concentrations of NaCl.[66] We found that the number of unexchanged hydrogens decreased by 15-19 in both acetylated and unmodified BCA II (Figure 7) as the concentration of added NaCl was increased from 0 M to 1 M; that is, sodium chloride led to an overall increase in the rate of H/D exchange for both acetylated and unmodified proteins. For unmodified BCA II, the number of unexchanged hydrogens retained after 80 min decreased by 14.7 ± 2.1 as the concentration of sodium chloride was increased from 0 M to 1 M. For rungs Ac(13)-(18), the number of unexchanged hydrogens decreased by 19.1 ± 4.0, 17.9 ± 3.4, 18.0 ± 2.7, 15.7 ± 3.1, 16.0 ± 2.7 and 14.1 ± 3.4. Interestingly, the number of unexchanged hydrogens in rungs Ac(14)-Ac(17) is, at 1.0 M NaCl, equivalent (within error) to the number of unexchanged hydrogens in unmodified BCA II at 0 M NaCl (Figure 7). These higher rungs of the charge ladder, however, still retain more unexchanged hydrogens than unmodified BCA II when both sets of proteins are at 0.1 or 1.0 M NaCl. This dissimilarity in the rate of exchange of the higher rungs of the charge ladder compared to the unmodified protein when both sets of proteins are in 1 M NaCl demonstrates that 1 M NaCl can not effectively screen all of the inter- or intramolecular electrostatic interactions that occur in the BCA II polypeptide and also with solvent. This result also suggests, however, that another electrostatic characteristic of the acetylated lysine residue—something other than its abolished charge—(i.e., perhaps hydrophobicity or inability to act as an acid catalyst) also contributes to the decreased rates of H/D exchange observed throughout the protein charge ladder.

Increasing Surface Hydrophobicity Does Not Affect the Rate of H/D Exchange of BCA II. In addition to neutralizing positive charges at the surface of BCA II, the acetylation of lysine also increases the surface hydrophobicity. The Hansch π-parameter (log P) for $-NH_3^+$ groups is log P = –2.12; and for $-NHCOCH_3$ groups, log P = –1.21.[67, 68] To test if the differences in the hydrogen exchange of each rung of the

charge ladder were due (entirely or in part) to increases in surface hydrophobicity, we prepared a perbutyrated derivative of BCA II (lys-ε-NHCO$(CH_2)_2CH_3$) by acylating all 18 lys-ε-NH_3^+ groups with butyric anhydride. The perbutyrated protein has 36 more –CH_2– groups on its surface than peracetylated BCA II; the perbutyrated protein will have a net charge similar to peracetylated BCA II, but a greater surface hydrophobicity (the Hansch parameter for NHCO$(CH_2)_2CH_3$ is not available, however, log P = 0.5 for –CH_3 and log P = 1.5 for –$(CH_2)_2CH_3$[67]).

We measured the hydrogen exchange of perbutyrated BCA II with LC-ESI-MS and compared its exchange profile with peracetylated BCA II (Figure 8). The global hydrogen exchange kinetics of these two derivatives of the same (we believe isostructural) protein were indistinguishable (Figure 8). Each set of data was fit with equation S1 (see supplemental material) and the resulting curves were superimposable (Figure 8). The similar rate of exchange demonstrates that surface hydrophobicity does not affect the kinetics of amide hydrogen exchange in BCA II; we conclude that increased surface hydrophobicity cannot explain the different rates of H/D exchange throughout the charge ladder.

Acetylation of Lysine Decreases the Thermostability of BCA II in Spite of Increasing its Protection from H/D Exchange. The rates of amide hydrogen exchange in folded proteins (and their chemical or genetic variants) *should* correlate inversely with the conformational stability as measured by unfolding with heat or chaotropic agent. This correlation has been observed on several occasions among homologous proteins and also among sets of protein variants prepared by site directed mutagenesis.[16, 17, 69] Equation 5 expresses the free energy of the transition from a *closed* to an *open* state (under EX2 conditions) as a function of the observed rate of H/D exchange.

$$\Delta G_{HD} = -RT \ln K_{op} = -RT \ln(k_{obs}/k_{int}) \qquad (5)$$

The rate of hydrogen exchange has, therefore, been used to measure the conformational stability of folded proteins.[15, 70, 71] We found, however, that the least thermally stable (and most highly charged) rungs of the charge ladder were more protected from H/D exchange than the more stable (and less charged) rungs. Our finding suggests that electrostatic factors such as net charge, or perturbations in the electrostatic environment

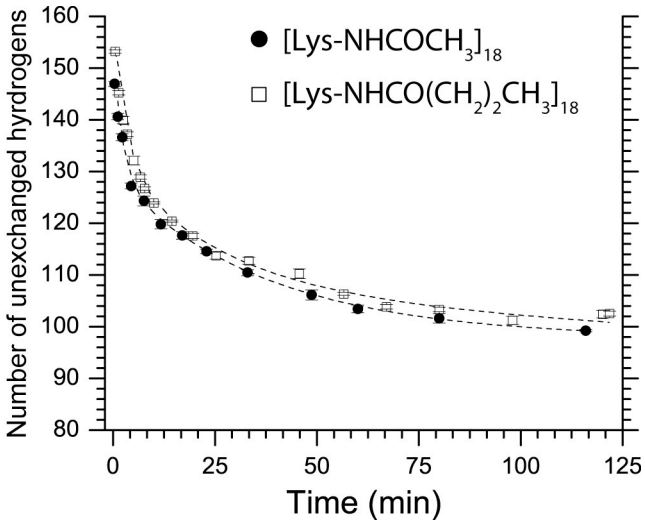

Figure 8. Increasing the surface hydrophobicity of BCA II has no effect upon the kinetics of hydrogen-deuterium exchange as measured by ESI-MS. The surface hydrophobicity of BCA II was varied, but the net charge was maintained, by attaching either 18 acetyl groups or 18 butanoyl groups. Both proteins exhibited similar protection from hydrogen exchange and nearly superimposable exchange profiles. Hydrogen exchange was monitored at 15 °C, pD 7.4, 10 mM PO_4^{3-}. Error bars represent the standard deviation of average mass values calculated from seven charge states for each protein.

of proteins that accompany post-translational modification, amino acid substitution, or ligand binding, can complicate the estimation of the conformational stability of a folded protein by measuring the rate of amide H/D exchange.

Conclusion

We have used protein charge ladders and mass spectrometry to quantify the effects of structural and electrostatic changes produced by conversion of lys-ε-NH_3^+ to lys-ε-$NHCOCH_3$ on the rate of H/D exchange in BCA II. Eliminating cationic sites on the surface of this protein by acetylation of its surface lysine-ε-NH_3^+ resulted in a decrease in the rate of amide H/D exchange that was linear in the number of lysine-ε-$NHCOCH_3$ groups formed. The neutralization of all 18 lysine residues by acetylation does not result in any significant changes in the 2° or 3° structure of BCA II (but did diminish the thermostability of the protein).

The electrostatic environment of an amide must therefore be considered along with its structural environment (e.g., its H-bonding and solvent accessibility) when interpreting the meaning of hydrogen exchange kinetics of folded proteins. The type of kinetic electrostatic effect—not structural effect—that we report in this paper might also arise, for example, from non-isoelectric perturbations such as amino acid substitution[72], post-translational modifications[73] (especially the acetylation of lysine or N-terminal –NH_3^+ [74, 75]) and the binding of charged small molecules[55, 76-78], oligonucleotides [79], or metal cofactors[80, 81] to proteins. For example, a recent paper has used mass spectrometry and H/D exchange to study how the acetylation of the N-terminus affects polypeptide chains that comprise the ribosomal stalk complex of *Escherichia coli.*[64] This paper reported that acetylation resulted in small increases in the number of amide hydrogens (i.e., between one and three) that were protected from exchange with buffer after 10 min in 90 % D_2O. Although the authors were reasonable in interpreting this decrease in the rate of H/D exchange to be caused by a tightening of the structure of the protein (and of the complex as a whole), we hypothesize that the decreased rate of H/D exchange occurred—at least in part—because of the same kinetic electrostatic effect that we observe with the protein charge ladder, and *not* because of a change in the structure or flexibility of the ribosomal stalk complex.

We hypothesize that the kinetic electrostatic effect that we observe arises from decreases in pH_{local} at the surface of the protein, and/or changes in the electrostatic environment near lysine residues that result in a reduction of the pK_a of nearby backbone amides. A definite, unambiguous identification of the specific residues that were protected from H/D exchange by acetylation in BCA II (which is necessary in order to best elucidate the mechanisms at work) is prevented by the lack of NMR peak assignments for BCA II. The rate of H/D exchange of model lysine compounds, however, suggests that the protection occurs at a backbone amide near the lysine residue. In addition to charge regulation, there are other plausible mechanistic explanations for why the acylation of ε-NH_3^+ decreases the rate of exchange in BCA II. The number of covalent bonds separating the ε-NH_3^+ group and backbone amide of lysine (e.g., six) is too great for the ε-NH_3^+ to exert a through-bond inductive effect on the backbone amide NH (that would reduce the pK_a of the amide NH); the ε-NH_3^+ group might, however, stabilize the anionic amide intermediate (e.g., –OCN^-–) that forms during base-catalyzed exchange of the amide hydrogen. We reiterate that the electrostatic effect that we observe—regardless of the exact molecular interactions that cause it—can not be entirely abolished (at least in the case of BCA II) by the addition of NaCl to solvent; the rates of H/D exchange of acetylated and non-acetylated BCA II are still different in the presence of 1 M NaCl (Figure 7). The future use of charge ladders of other stable proteins whose NMR structures have been determined (i.e., rubredoxin or superoxide dismutase-1) should provide greater insight into the exact mechanism by which surface electrostatics affect the hydrogen exchange of folded proteins.

This study has, nevertheless, shown that protein charge ladders offer a unique tool to use in understanding the structural and electrostatic factors that govern the rate of hydrogen exchange in folded proteins (and that have, so far, been intractably difficult to explore experimentally and hence largely overlooked). A more complete theory of hydrogen exchange in folded polypeptides will hopefully explain some of the surprising and sometimes confusing results that accumulated for over 50 years in biochemistry and in organic and polymer chemistry.[19-22, 35-37, 82, 83]

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Supporting Information Available: Additional experimental details, including mass spectrometric experiments and kinetic analysis of mass spectrometric data, and additional data, including tabulated values of mass and kinetic parameters, NMR spectra, and kinetic exchange profiles for all 19 rungs of the BCA II charge ladder. This material is available free of charge via the Internet at http://pubs.acs.org.

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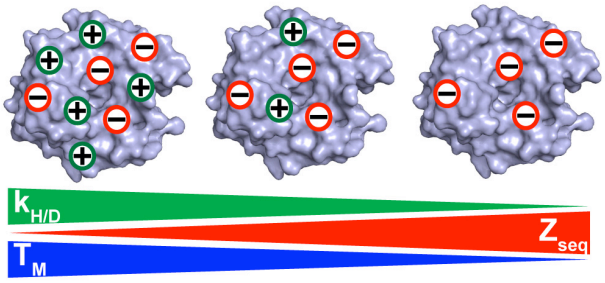

References

1. Gao, J.; Mammen, M.; Whitesides, G. M., *Science* **1996,** 272, 535-7.
2. Gitlin, I.; Carbeck, J. D.; Whitesides, G. M., *Angew. Chem. Int. Ed. Engl.* **2006,** *45*, 3022-60.
3. Gitlin, I.; Gudiksen, K. L.; Whitesides, G. M., *J. Phys. Chem. B* **2006,** *110*, 2372-7.
4. Negin, R. S.; Carbeck, J. D., *J. Am. Chem. Soc.* **2002,** *124*, 2911-6.
5. Gudiksen, K. L.; Gitlin, I.; Moustakas, D. T.; Whitesides, G. M., *Biophys. J.* **2006,** *91*, 298-310.
6. Krishnamurthy, V. M.; Kaufman, G. K.; Urbach, A. R.; Gitlin, I.; Gudiksen, K. L.; Weibel, D. B.; Whitesides, G. M., *Chem. Rev.* **2008,** *108*, 946-1051.

7. Menon, M. K.; Zydney, A. L., *Anal. Chem.* **2000,** *72*, 5714-7.
8. Snyder, P. W.; Shaw, B. F.; Butte, M. J.; Moustakas, D. T.; Whitesides, G. M., Manuscript in Preparation.
9. The mass spectrometric methods that we used in this research did not involve fragmenting the protein with proteolysis prior to analysis with mass spectrometry. We, therefore, measured the H/D exchange of the entire polypeptide and refer to this exchange as 'global'.
10. Englander, S. W.; Krishna, M. M., *Nat. Struct. Biol.* **2001,** *8*, 741-2.
11. Hvidt, A.; Linderstrom-Lang, K., *Biochim. Biophys. Acta* **1954,** *14*, 574-5.
12. Wagner, G.; Wuthrich, K., *J. Mol. Biol.* **1979,** *134*, 75-94.
13. Wagner, G.; Wuthrich, K., *Nature* **1978,** *275*, 247-8.
14. Dobson, C. M., *Semin. Cell. Dev. Biol.* **2004,** *15*, 3-16.
15. Huyghues-Despointes, B. M.; Langhorst, U.; Steyaert, J.; Pace, C. N.; Scholtz, J. M., *Biochemistry* **1999,** *38*, 16481-90.
16. Rodriguez, J. A.; Shaw, B. F.; Durazo, A.; Sohn, S. H.; Doucette, P. A.; Nersissian, A. M.; Faull, K. F.; Eggers, D. K.; Tiwari, A.; Hayward, L. J.; Valentine, J. S., *Proc. Natl. Acad. Sci. U S A* **2005,** *102*, 10516-21.
17. Wagner, G.; Wuthrich, K., *J. Mol. Biol.* **1979,** *130*, 31-7.
18. Englander, S. W., *Annu. Rev. Biophys. Biomol. Struct.* **2000,** *29*, 213-38.
19. Hvidt, A.; Corret, R., *J. Am. Chem. Soc.* **1970,** *92*, 5546-5550.
20. Perrin, C.; Chen, J.; Ohta, B., *J. Am. Chem. Soc.* **1999,** *121*, 2448-2455.
21. Scarpa, J.; Mueller, D.; Klotz, I., *J. Am. Chem. Soc.* **1967,** *89*, 6024-6030.
22. Anderson, J. S.; Hernandez, G.; Lemaster, D. M., *Biochemistry* **2008,** *47*, 6178-88.
23. Bai, Y., *Chem. Rev.* **2006,** *106*, 1757-68.
24. Maity, H.; Lim, W. K.; Rumbley, J. N.; Englander, S. W., *Protein Sci.* **2003,** *12*, 153-60.
25. Connelly, G. P.; Bai, Y.; Jeng, M. F.; Englander, S. W., *Proteins* **1993,** *17*, 87-92.
26. Bai, Y.; Sosnick, T. R.; Mayne, L.; Englander, S. W., *Science* **1995,** *269*, 192-7.
27. One example of this type of slow exchange is the amide hydrogen on Leucine 98 in native cytochrome c; this hydrogen exchanges on a timescale of ~100 years at neutral pH.
28. Hvidt, A.; Nielsen, S. O., *Adv Protein Chem.* **1966,** *21*, 287-386.
29. Molday, R. S.; Englander, S. W.; Kallen, R. G., *Biochemistry* **1972,** *11*, 150-8.
30. Bai, Y.; Milne, J. S.; Mayne, L.; Englander, S. W., *Proteins* **1993,** *17*, 75-86.
31. Wagner, G., *Biochem. Biophys. Res. Comm.* **1980,** *97*, 614-20.
32. Roder, H.; Wagner, G.; Wuthrich, K., *Biochemistry* **1985,** *24*, 7396-407.
33. Kim, K. S.; Woodward, C., *Biochemistry* **1993,** *32*, 9609-13.
34. Woodward, C.; Barbar, E.; Carulla, N.; Battiste, J.; Barany, G., *J Mol Graph Model* **2001,** *19*, 94-101.
35. Miller, D. W.; Dill, K. A., *Protein Sci.* **1995,** *4*, 1860-73.
36. Forsyth, W.; Robertson, A., *J. Am. Chem. Soc.* **1996,** *118*, 2694-2698.
37. Radkiewicz, J. L.; Zipse, H.; Clarke, S.; Houk, K. N., *J Am Chem Soc* **2001,** *123*, 3499-506.
38. High ionic strength (> 100 mM) is nevertheless more physiologically relevant than low ionic strength (< 100 mM).

39. Hernandez, G.; Anderson, J. S.; LeMaster, D. M., *Biochemistry* **2009,** *48*, 6482-94.
40. Anderson, J. S.; Hernandez, G.; LeMaster, D. M., *Biophys. Chem.* **2009,** *141*, 124-30.
41. Hernandez, G.; Anderson, J. S.; LeMaster, D. M., *ChemBioChem* **2008,** *9*, 768-78.
42. Dempsey, C. E., *Biochemistry* **1986,** *25*, 3904-11.
43. Matthew, J. B.; Richards, F. M., *J. Biol. Chem.* **1983,** *258*, 3039-44.
44. Christoffersen, M.; Bolvig, S.; Tuchsen, E., *Biochemistry* **1996,** *35*, 2309-15.
45. Kim, P. S.; Baldwin, R. L., *Biochemistry* **1982,** *21*, 1-5.
46. Goldfarb, N. E.; Lam, M. T.; Bose, A. K.; Patel, A. M.; Duckworth, A. J.; Dunn, B. M., *Biochemistry* **2005,** *44*, 15725-33.
47. Leikina, E.; Ramos, C.; Markovic, I.; Zimmerberg, J.; Chernomordik, L. V., *EMBO J.* **2002,** *21*, 5701-10.
48. Shaw, A. K.; Pal, S. K., *J. Photochem. Photobiol. B* **2008,** *90*, 187-97.
49. Shaw, B. F.; Schneider, G. F.; Bilgicer, B.; Kaufman, G. K.; Neveu, J. M.; Lane, W. S.; Whitelegge, J. P.; Whitesides, G. M., *Protein Sci.,* **2008,** *17*, 1446-55.
50. Pervushin, K.; Riek, R.; Wider, G.; Wuthrich, K., *Proc. Natl. Acad. Sci. U S A* **1997,** *94*, 12366-71.
51. Ruben, D. J.; Bodenhausen, G., *Chem. Phys. Lett.* **1980,** *69*, 185-189.
52. Bax, A.; Ikura, M.; Kay, L. E.; Barbato, G.; Spera, S., *Ciba. Found. Symp.* **1991,** *161*; discussion 119-35.
53. Goddard, T.; Kneller, D. *SPARKY 3 - NMR Assignment and Integration Software. University of California, San Francisco*, 2006.
54. Burton, R. E.; Hunt, J. A.; Fierke, C. A.; Oas, T. G., *Protein Sci.* **2000,** *9*, 776-85.
55. Schneider, G. F.; Shaw, B. F.; Lee, A.; Carillho, E.; Whitesides, G. M., *J. Am. Chem. Soc.* **2008,** *130*, 17384-93.
56. Proteolysis of BCA II under conditions where H/D exchange is quenched could provide low resolution information about the rate of specific amino acids in CA II; the resolution provided by these methods is limited by the size of the proteolytic fragment that is generated by reactions with pepsin.
57. To illustrate this point, consider for example a 100 residue protein and a 150 residue protein that both incorporate 75 amide deuterons after 80 min in D_2O. Although both proteins have identical rates of deuterium incorporation, the 150 residue protein is, in fact, more protected from hydrogen exchange, or more "structured" than the 100 residue protein because the 150 residue protein has 75 residues that have not exchanged with solvent (the 100 kDa protein has only 25).
58. Shaw, B. F.; Durazo, A.; Nersissian, A. M.; Whitelegge, J. P.; Faull, K. F.; Valentine, J. S., *J. Biol. Chem.* **2006,** *281*, 18167-76.
59. Jonasson, P.; Kjellsson, A.; Sethson, I.; Jonsson, B. H., *FEBS Lett.* **1999,** *445*, 361-5.
60. Wang, L.; Smith, D. L., *Curr. Protoc. Protein Sci.* **2002,** Chapter 17, Unit 17 6.
61. The indole-H of deeply buried tryptophan residues in BCA II may not exchange rapidly with solvent. An analysis of the hydrogen exchange properties of tryptophan residues in human CA II, with NMR, revealed that several solvent-exposed Trp residues (i.e., Trp5, 16, and 245) undergo H/D exchange rapidly

(e.g., $t_{1/2}$<< 20 min) while buried residues (i.e., Trp97) did not exchange with solvent (e.g., $t_{1/2}$>> 20 min) unless the protein was unfolded with guanidinium hydrochloride. Some tryptophan residues, therefore, might be mistaken as amide hydrogen when using mass spectrometric methods to measure H/D exchange of BCA II.

62. Colton, I. J.; Anderson, J. R.; Gao, J. M.; Chapman, R. G.; Isaacs, L.; Whitesides, G. M., *J. Am. Chem. Soc.* **1997,** *119*, 12701-12709.
63. An alignment of amino acid sequences of human and bovine CA II (using Clusta1X software) revealed a sequence homology of 79 %. The pI of HCA II is 7.6 (the pI of BCA II =5.9). Previous analysis of HCA II and BCA II with X-ray crystallography show that the two proteins have the same over-all fold and nearly identical structures. Approximately 1715 atoms could be aligned from each crystal structure of HCA II and BCA II (HCA II contains 4083 atoms; BCA II contains 4048) and the root mean squared deviation (RMS) for these 1715 aligned atoms was 0.448 Å.
64. Cavanagh, J.; Fairbrother, W. J.; Palmer III, A. G.; France, A.; Skelton, N. J., *Protein NMR Spectroscopy: Principles and Practice*. Academic Press: 2007.
65. Wuthrich, K., *J. Biol. Chem.* **1990,** *265*, 22059-62.
66. The solutions of charge ladder must be diluted considerably (i.e., 100 fold) when 1 M NaCl is present before injection into the mass spectrometer, in addition to the initial 10-fold dilution into D_2O from H_2O. Achieving an appropriate final concentration of protein for analysis with ESI-MS is more convenient with a partial ladder that contains only 3-4 abundant rungs rather than a full charge ladder that contains 19.
67. Hansch, C.; Coats, E., *J. Pharm. Sci.* **1970,** *59*, 731-43.
68. Hansch, C.; Steward, A. R., *J. Med. Chem.* **1964,** *7*, 691-4.
69. Wagner, G.; Wuthrich, K., *J. Mol. Biol.* **1982,** *160*, 343-61.
70. Huyghues-Despointes, B. M.; Scholtz, J. M.; Pace, C. N., *Nat. Struct. Biol.* **1999,** *6*, 910-2.
71. Mullins, L. S.; Pace, C. N.; Raushel, F. M., *Protein Sci.* **1997,** *6*, 1387-95.
72. Chitta, R. K.; Rempel, D. L.; Grayson, M. A.; Remsen, E. E.; Gross, M. L., *J. Am. Soc. Mass. Spectrom.* **2006,** *17*, 1526-34.
73. Jones, D. D.; Stott, K. M.; Howard, M. J.; Perham, R. N., *Biochemistry* **2000,** *39*, 8448-59.
74. Gordiyenko, Y.; Deroo, S.; Zhou, M.; Videler, H.; Robinson, C. V., *J. Mol. Biol.* **2008,** *380*, 404-14.
75. Szewczuk, Z.; Konishi, Y.; Goto, Y., *Biochemistry* **2001,** *40*, 9623-30.
76. Wildes, D.; Marqusee, S., *Protein Sci.* **2005,** *14*, 81-8.
77. Marques, M. R.; Vaso, A.; Neto, J. R.; Fossey, M. A.; Oliveira, J. S.; Basso, L. A.; dos Santos, D. S.; de Azevedo Junior, W. F.; Palma, M. S., *Biochemistry* **2008,** *47*, 7509-22.
78. Das, R.; Esposito, V.; Abu-Abed, M.; Anand, G. S.; Taylor, S. S.; Melacini, G., *Proc. Natl. Acad. Sci. U S A* **2007,** *104*, 93-8.
79. Sperry, J. B.; Wilcox, J. M.; Gross, M. L., *J. Am. Soc. Mass Spectrom.* **2008,** *19*, 887-90.

80. Potter, S. Z.; Zhu, H.; Shaw, B. F.; Rodriguez, J. A.; Doucette, P. A.; Sohn, S. H.; Durazo, A.; Faull, K. F.; Gralla, E. B.; Nersissian, A. M.; Valentine, J. S., *J. Am. Chem. Soc.* **2007,** *129*, 4575-83.
81. Ferguson, P. L.; Pan, J.; Wilson, D. J.; Dempsey, B.; Lajoie, G.; Shilton, B.; Konermann, L., *Anal. Chem.* **2007,** *79*, 153-60.
82. Brorsson, A. C.; Lundqvist, M.; Sethson, I.; Jonsson, B. H., *J. Mol. Biol.* **2006,** *357*, 1634-46.
83. Perrin, C., *Acc. Chem. Res.* **1989,** *22*, 268-275.